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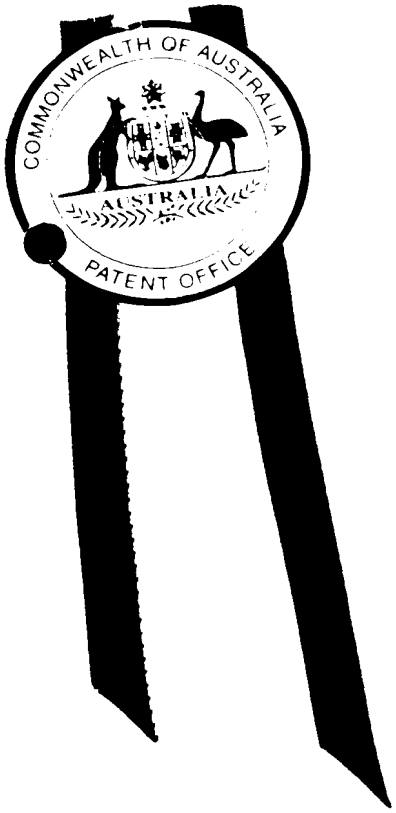

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AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

"A METHOD OF TREATMENT AND AGENTS USEFUL FOR SAME"

The invention is described in the following statement:-

metastasising to lymph nodes and other parts of the body. Survival rates are significantly improved where the disease is detected and treated early.

The aetiology of colorectal cancer is complex and appears to involve interactions between inherited susceptibility and environmental factors. Recognition of the genetic component of colorectal cancer is growing. Mutations are present as inherited germline defects or arise in somatic cells secondary to environmental insult. There are two main inherited predisposition syndromes: Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC); the remaining cases are attributed to so-called sporadic colorectal cancer. FAP and HNPCC contribute to approximately 1% and 4%, respectively, of all colorectal cancers and a strong family history of bowel cancer in first-degree relatives is obtained in another 10 - 15% of patients.

However, in the vast majority of patients the aetiology of large bowel cancer remains unknown. Most colon cancer arises within pre-existing benign precursor lesions or adenomas. Adenomas are classified by histological architecture as tubular, tubulovillous or villous. Villous change is associated with a higher malignant potential, as are large and high-grade epithelial dysplasia. Environmental risk factors for development of colorectal cancer include diets low in fibre and vegetables and high in fat, red meat and alcohol and cigarette smoking which may induce mutations in somatic cells.

Studies have shown that persistent genetic instability and accumulation of mutations in several genes that are mainly concerned with cell growth or DNA repair, may be critical for the development of all colorectal cancers. For example, a normal

including integrins, cadherins, the immunoglobulin superfamily, hyaluronate receptors, and mucins (Agrez, 1996.) In general, these cell surface molecules mediate both cell-cell and cell-matrix binding, the latter involving attachment of tumour cells to extracellular scaffolding molecules such as collagen, fibronectin and laminin. It is now clear that multiple and varied cell adhesion receptors exist on colon cancer cells at any one time and an understanding of the role of individual receptors in promoting growth and spread of colon cancer is only just beginning to be elucidated.

Of all the families of cell adhesion molecules, the best-characterised at the present time is the family known as integrins. Integrins are involved in several fundamental processes including leucocyte recruitment, immune activation, thrombosis, wound healing, embryogenesis, virus internalisation and tumourigenesis. Integrins are transmembrane glycoproteins consisting of an alpha (α) and beta (β) chain in close association that provide a structural and functional bridge between extracellular matrix molecules and cytoskeletal components with the cell. The integrin family comprises 17 different α and 8 β subunits and the $\alpha\beta$ combinations are subsumed under 3 subfamilies.

Excluding the leucocyte integrin subfamily that is designated by the $\beta 2$ nomenclature, the remaining integrins are arranged into two major subgroups, designated $\beta 1$ and αv based on sharing common chains.

In the $\beta 1$ subfamily, the $\beta 1$ chain combines with any one of nine α chain members ($\alpha 1$-9), and the α chain which associates with $\beta 1$ determines the matrix-binding specificity of that receptor. For example, $\alpha 2\beta 1$ binds collagen and laminin, $\alpha 3\beta 1$ binds collagen, laminin and fibronectin, and $\alpha 5\beta 1$ binds fibronectin. In the αv subfamily of receptors, the abundant and promiscuous αv chain combines with any one of five β

between these cytoplasmic domains with regard to their signalling capacity have not yet been established.

Ligation of integrins by their extracellular matrix protein ligands induces a cascade of intracellular signals that include tyrosine phosphorylation of focal adhesion kinase, increases in intracellular Ca^{2+} levels, inositol lipid synthesis, synthesis of cyclins and expression of immediate early genes. In contrast, prevention of integrin-ligand interactions suppresses cellular growth or induces apoptotic cell death (Meredith et al, 1993; Montgomery et al, 1994; Brooks et al, 1994; Varner et al, 1995; Boudreau et al, 1995). Thus, integrins play roles in a number of cellular processes that impact on the development of tumours, including the regulation of proliferation and apoptosis.

The integrin β6 subunit was first identified in cultured epithelial cells as part of the αvβ6 heterodimer, and the αvβ6 complex was shown to bind fibronectin in an arginine-glycine-aspartate (RGD)-dependent manner in human pancreatic carcinoma cells (Sheppard et al, 1990; Busk et al, 1992). The β6 subunit is composed of 788 amino acids and shares 34 - 51% sequence homology with other β integrin subunits β1 - β5. The β6 subunit also contains 9 potential glycosylation sites on the extracellular domain (Sheppard et al, 1990). The cytoplasmic domain differs from other β subunits in that it is composed of a 41 amino acid region that is highly conserved among integrin β subunits, and a unique 11 amino acid carboxy-terminal extension. The 11 amino acid extension has been shown not to be necessary for localisation of β6 to focal contacts; in fact, its removal appears to increase receptor localisation. However, removal of any of the three conserved regions previously identified as important for the localisation of β1

phosphatidylinositol-4, 5-biphosphate (P_1P_2) synthesis. The second category of integrin signalling is 'collaborative signalling', in which integrin-mediated cell adhesion modulates signalling events initiated through other types of receptors, particularly receptor tyrosine kinases that are activated by polypeptide growth factors (Howe et al, 1998). In all cases, however, integrin-mediated adhesion seems to be required for efficient transduction of signals into the cytosol or nucleus.

MAP kinases behave as a convergence point for diverse receptor-initiated signalling events at the plasma membrane. The core unit of MAP kinase pathways is a three-member protein kinase cascade in which MAP kinases are phosphorylated by MAP kinase kinases (MEKs) which are in turn phosphorylated by MAP kinase kinase kinases (e.g. Raf-1) (Garrington and Johnson, 1999). Amongst the 12 member proteins of the MAP kinase family are the extracellular signal-regulated kinases (ERKs) (Boulton et al, 1991) activated by phosphorylation of tyrosine and threonine residues (Payne et al, 1991) which is the type of activation common to all known MAP kinase isoforms. ERK 1/2 (44kD and 42kD MAPks, respectively) share 90% amino acid identity and are ubiquitous components of signal transduction pathways (Boulton et al, 1991). These serine/threonine kinases phosphorylate and modulate the function of many proteins with regulatory functions including other protein kinases (such as $p90^{rsk}$) cytoskeletal proteins (such as microtubule-associated phospholipase A_2), upstream regulators (such as the epidermal growth factor receptor and Ras exchange factor) and transcription factors (such as C-Myc and Elk-1).

MAP kinases can be activated through non-receptor tyrosine kinases such as focal adhesion kinase (FAK), cytoplasmic tyrosine kinase (pp60 c-src) (Schlaepfer and

(PKC) isoforms and is thought to be due to inactivation of a MAP kinase inhibitor (Grammer and Blenis, 1997).

Although the mechanism by which PKC regulates integrin function is not known, PKC has been shown to regulate integrin-induced activation of the MAP kinase pathway upstream of Shc. For example, PKC inhibition has been shown to inhibit ERK2 activation by fibronectin receptors without any effect on integrin-induced FAK or paxillin tyrosine phosphorylation (Miranti et al, 1999). Hence, MAP kinase activation is more complicated than a simple linear pathway, and the mechanistic basis for the commonly observed integrin-mediated activation of MAP kinases remains controversial.

Various intracellular proteins may be linked directly or spatially to integrin cytoplasmic domains. Direct interactions have been identified between cytoskeletal proteins such as α-actinin and talin and β1 and β3 integrin tails (Horwitz et al, 1986; Otey et al, 1990; Knezevic et al, 1996; Pfaff et al, 1998). A direct association between FAK and the β1 integrin tail has been suggested based on *in vitro* β1 peptide studies, but this remains to be confirmed (Schaller et al, 1995). More recently, the cytoplasmic domain of the α4 subunit has been found to be physically associated with the signalling adaptor protein paxillin in Jurkat T cells, and this binding event regulates the kinetics of FAK tyrosine phosphorylation (Liu et al, 1999).

Direct integrin links with the intracellular calcium-binding protein, calreticulin, and integrin-linked kinase (ILK) (Hannigan et al, 1996) have been shown to regulate "inside-out" integrin signalling. For example, calreticulin has been shown to bind to α chain cytoplasmic domains (Rojiani et al, 1991) and modify α2β1 integrin activation by phorbol esters and anti-integrin antibodies (Coppolino et al, 1995). Newly identified

Summary Of The Invention

Broadly stated, the present invention relates to modulation of integrin expression in neoplastic cells to inhibit the growth of the cells, and the surprising finding that members of the mitogen activated protein (MAP) kinase family associate with the cytoplasmic domain of an integrin molecule. It is believed that no member of the MAP kinase family has previously been found to directly associate with any integrin or for that matter, with any transmembrane protein. The identification of this functional relationship permits the rational design of agents for therapeutically or prophylactically modulating cellular activity mediated by the MAP kinase and integrin interaction.

In an aspect of the present invention there is provided an agent capable of inhibiting binding of a MAP kinase with an integrin.

Typically, the agent will be capable of binding with a binding site on the MAP kinase that binds to a binding domain on the integrin for the MAP kinase. Alternatively, the agent may be capable of binding to the binding domain on the integrin for the MAP kinase or other site on the integrin such that inhibition of binding of the MAP kinase to the integrin is thereby caused.

The agent may be provided either isolated or for instance, coupled to another molecule for facilitating transport of the agent into a cell.

In another aspect of the present invention there is provided an isolated polypeptide capable of binding with a binding site on a MAP kinase which binding site binds with a binding domain on an integrin for the MAP kinase, or a homolog, analog, variant or derivative of the polypeptide,with the proviso that the polypeptide is other than a full length integrin subunit or a β6(770t) or β6(777t) deletion mutant.

In still another aspect of the present invention there is provided a fusion protein incorporating a polypeptide of the invention or a homolog, analog or variant of the polypeptide.

In a further aspect of the present invention there is provided a fusion protein incorporating a fragment of the invention or a homolog, analog or variant of the fragment.

Typically, the polypeptide or fragment will be coupled to a carrier polypeptide for facilitating entry of the fusion protein into a cell. Preferably, the carrier polypeptide will be penetatin.

In another aspect of the present invention there is provided an isolated nucleic acid sequence encoding a polypeptide of the invention or a homolog, analog, or variant of the polypeptide.

In yet another aspect of the invention there is provided an isolated nucleic acid sequence encoding a fragment of the invention or a homolog, analog, or variant of the fragment.

In another aspect of the invention there is provided a nucleic acid sequence encoding an integrin subunit with a mutagenised binding domain for a MAP kinase or in which the binding domain is deleted,wherein capability of the integrin subunit to bind with the MAP kinase is thereby reduced, or a homolog, analog, or variant of the integrin subunit, with the proviso that the integrin subunit is other than a β6 Δ746-764 deletion mutant.

In a still further aspect of the present invention there is provided an isolated nucleic acid sequence encoding a fusion protein of the invention.

In another aspect of the present invention there is provided a method of isolating a MAP kinase from a sample, comprising:

(a) contacting an antibody of the invention immobilised on a solid support with the sample under conditions suitable for binding of the antibody to the MAP kinase;

(b) eluting the MAP kinase from the support; and

(c) collecting the eluted MAP kinase.

In still another aspect of the present invention there is provided a method of isolating a MAP kinase from a sample utilising a molecule immobilised on a solid support and which is capable of binding to a binding site on the MAP kinase which binding site binds with a binding domain on an integrin for the MAP kinase, comprising:

(a) contacting the molecule immobilised on the solid support with the sample under conditions suitable for binding of the MAP kinase to the molecule;

(b) eluting the MAP kinase from the solid support;

(c) collecting the eluted MAP kinase

The molecule may be the integrin, or a fusion protein, a polypeptide, or a fragment of the invention to which the MAP kinase is capable of binding, or for instance an analog, homolog, variant or derivative of the polypeptide or fragment.

In yet another aspect of the present invention there is provided a MAP kinase isolated by a method of the invention.

Rather than isolating the MAP kinase from a sample, the MAP kinase may be immobilised on a solid support and used to isolate the molecule from a sample, and all such methods are also expressly encompassed as is the molecule when isolated in this way.

integrin may be used. Alternatively, the testing or assaying may comprise exposing the MAP kinase to the agent to enable binding of the agent to the MAP kinase to occur either in the presence of the integrin or other molecule capable of binding with the binding site on the MAP kinase, or prior to the addition of the integrin or molecule.

In still another aspect of the present invention there is provided a method of screening for an agent capable of binding to a binding domain on an integrin for a MAP kinase, comprising:

(a) testing a number of agents for ability to bind to the binding domain on the integrin for the MAP kinase; and

(b) determining if any said agent is capable of binding to the binding domain on the integrin on the basis of the testing.

In yet another aspect of the invention there is provided a method of screening for an agent capable of binding to a binding domain on an integrin for a MAP kinase, comprising:

(a) testing a number of agents for ability to bind to the integrin;

(b) selecting an agent or agents identified as being able to bind to the integrin on the basis of the testing; and

(c) utilising the selected said agent or agents in an assay for indicating whether the or any of the selected said agents is capable of binding to the binding domain on the integrin for the MAP kinase.

In another aspect of the invention there is provided a method of evaluating whether an agent is capable of inhibiting binding of a MAP kinase to a binding domain on an integrin for the MAP kinase, comprising:

into a vector as described herein. Alternatively, the nucleic acid sequence may be joined to a carrier molecule for facilitating entry of the nucleic acid sequence into a target cell.

In a further aspect of the present invention there is provided a method of modulating activity of a cell, comprising:

transfecting the cell with a nucleic acid sequence encoding an integrin subunit for being expressed by the cell, wherein the integrin subunit has a mutagenised binding domain for a MAP kinase or in which the binding domain is deleted, or a homolog, analog or variant of the integrin subunit.

In yet another aspect of the present invention there is provided a method of modulating activity of a cell, comprising:

transfecting the cell with a nucleic acid encoding a polypeptide for being expressed by the cell wherein the polypeptide is capable of inhibiting binding of a MAP kinase with a binding domain on an integrin for the MAP kinase, or a homolog, analog or variant of the polypeptide.

Preferably, the polypeptide will be capable of binding with the binding site on the MAP kinase which binding site binds to the binding domain on the integrin.

In still another aspect of the invention there is provided a method of modulating activity of a cell, comprising causing the expression of an integrin to which a MAP kinase is able to bind to be down-regulated.

Preferably, down-regulation of the expression of the integrin is achieved using an antisense nucleic acid sequence that inhibits expression of the gene encoding the integrin. The antisense nucleic acid sequence may be administered to the cell or

In a further aspect of the present invention there is provided a method of prophylaxis or treatment of a disease or condition in a mammal wherein modulation of cell activity is desirable, comprising:

administering to the mammal an effective amount of a nucleic acid sequence encoding an integrin subunit for being expressed, wherein the integrin subunit has a mutagenised binding domain for a MAP kinase or in which the binding domain is deleted, or a homolog, analog or variant of the integrin subunit.

In another aspect of the invention there is provided a method of prophylaxis or treatment of a disease or condition in a mammal wherein modulation of cell activity is desirable, comprising:

administering to the mammal an effective amount of a nucleic acid of the invention capable of causing the expression of an integrin to which a MAP kinase is able to bind to be down regulated.

In still another aspect of the present invention there is provided a method of prophylaxis or treatment of a disease or condition in a mammal wherein modulation of cell activity is desirable, comprising:

administering to the mammal an effective amount of a nucleic acid sequence encoding a polypeptide for being expressed, wherein the polypeptide is capable of inhibiting binding of a MAP kinase with a binding domain of an integrin for the MAP kinase to thereby cause down-regulation of MAP kinase integrin binding, or a homolog, analog or variant of the polypeptide.

In another aspect of there is provided a method of treatment or prophylaxis of a disease or condition in a mammal, wherein said condition is responsive to an agent of the

Usually, the MAP kinase will be selected from the group consisting of an extracellular signal-regulated kinase (ERK) and a JNK MAP kinase. Preferably, the MAP kinase is ERK2 or JNK-1. Most preferably, the MAP kinase is ERK2.

The mammal may be any mammal treatable with a method of the invention. For instance, the mammal may be a member of the bovine, porcine, ovine or equine families, a laboratory test animal such as a mouse, rabbit, guinea pig, a cat or dog, a primate or a human being. Preferably, the mammal will be a human being.

The term "modulating" is to be taken as reference to down-regulating the activity of the cell or the functional activity of the integrin. Reference to "down-regulating" should be understood to include preventing, reducing or otherwise inhibiting one or more aspects of the activity of the cell or the functional activity of the integrin molecule or the MAP kinase

In the broadest sense, the term "integrin" unless otherwise specified, is to be taken to encompass an integrin family member or a homolog, derivative, variant or analog of an integrin subunit, or an integrin family member incorporating at least one such homolog, derivative, variant or analog of an integrin subunit. Usually, the integrin will be a member of the αv subfamily. Preferably, the integrin is or incorporates the integrin β6 subunit.

By "binding domain" is meant the minimum length of contiguous amino acid sequence required for binding by the MAP kinase. By "core amino acid sequence" is meant regions or amino acids of the binding domain that directly participate in the binding as distinct from any amino acids that do not directly participate in the binding interaction with the MAP kinase. Typically, the core amino acid sequence of the binding

such biological function. Indeed, it is not necessary that an analog have amino acid sequence homology, and an analog may not be proteinaceous at all. An analog may for instance be a mimetic of a molecule.

By the term "variant" is meant an isoform of an integrin subunit, an integrin subunit encoded by an allelic variant of a gene for an integrin subunit, a naturally occurring mutant form of a gene for an integrin subunit, or an integrin subunit or polypeptide having an amino acid sequence that differs in one or more amino acids but which retains one or more aspects of desired characteristic biological function. This may be achieved by the addition of one or more amino acids to an amino acid sequence, deletion of one or more amino acids from an amino acid sequence and/or the substitution of one or more amino acids with another amino acid or amino acids. Inversion of amino acids and any other mutational change that results in alteration of an amino acid sequence are also encompassed. A variant may be prepared by introducing nucleotide changes in a nucleic acid sequence that encodes for an integrin subunit or amino acid sequence such that the desired amino acid changes are achieved upon expression of the mutagenised nucleic acid sequence, or for instance by synthesising an integrin subunit or amino acid sequence incorporating the desired amino acid changes, both of which possibilities are well within the capability of the skilled addressee.

Substitution of an amino acid may involve a conservative or non-conservative amino acid substitution. By conservative amino acid substitution is meant replacing an amino acid residue with another amino acid having similar stereochemical properties (eg. structure, charge, acidity or basicity characteristics) and which does not substantially effect conformation or the desired aspect or aspects of characteristic biological function.

inclusive sense as opposed to an exclusive or exhaustive sense: that is to say, in the sense of "including, but not limited to".

The features and advantages of the present invention will become further apparent from the following detailed description of preferred embodiments and the accompanying drawings.

Brief Description Of The Accompanying Drawings

Figure 1: Surface biotinylation and immunoprecipitation of integrin subunits β5 and β6 in HT29 colon cancer cells stably transfected with either vector alone (mock transfectants) or antisense β6 construct;

Figure 2: Amplification of β6 and glyceraldehyde dehydrogenase (GAPDH) mRNA: Ethidium-stained agarose gels with amplification products following RT-PCR from total RNA extracted from transfected HT29 and WiDr cell lines. Equal amounts of PCR product obtained from RT-PCR reactions were loaded on each lane and the β6 (141 basepairs) and GAPDH (216 basepairs) bands are indicated (WT, wild-type; S, sense β6; A/S, antisense β6; mock, vector alone);

Figure 3: Non-transfected HT29 cells (wild) and cells transfected with vector alone (mock), sense β6 and antisense β6 analysed by FACScan for expression of the β6 subunit. White and black histograms represent cells stained in the absence and presence, respectively, of mAb E7P6 (anti-β6);

Figure 4: WiDr cells transfected with vector alone (mock) or antisense β6 and analysed by FACScan for expression of the β6 subunit. White and black histograms represent cells stained in the absence and presence, respectively, of mAb E7P6 (anti-β6);

the left hand lane. (B) β6 immunoprecipitates (mAb R6G9) from equal protein loads of the cell lysates in (A) probed with anti-ERK mAb (E10). Purified phosphorylated ERK2 is shown in the left hand lane:

Figure 12: β6-bound ERK shown for the high and low SW480 β6-expressing clones by probing β6 immunoprecipitates with anti-ERK mAb (E10) against phosphorylated forms of ERK1/2. Purified, phosphorylated ERK2 is shown in the left hand lane:

Figures 13(A) and 13(B): (A) Surface biotinylation of WiDr wild-type cells and β6 immunodepletion of the cell lysates by three successive rounds of β6 and β5 immunoprecipitations using mAb R6G9 and P1F6, respectively or control mAb (IgG2A). The β6 and partner αv bands are arrowed. (B) β6-immunodepleted WiDr cell lysates after 3 successive rounds of β6-immunoprecipitations probed with anti-ERK mAb SC-1647 recognising both phosphorylated and non-phosphorylated forms of ERK1/2 and compared with non-β6 immunodepleted lysates and control lysates sequentially immunoprecipitated 3 times with either isotype matched control mAb (IgG2A) or mAb P1F6 (anti-β5):

Figure 14: Non-transformed (HaCaT) and Ras-transformed (HaRas) human keratinocytes: β6 immunoprecipitation and ERK western blots probed with monoclonal antibody E10 (against Phosphorylated ERK 1/2) and monoclonal antibody SC1647 (against total ERK 1/2), respectively.

Figure 15(A) and 15(B): (A) Depletion of β6 with sequential rounds of ERK immunodepletion of cell lysates using anti-ERK mAb (SC-1647). (B) MAP kinase activity in cell lysates before and after 5 rounds of sequential β6 immunodepletion from

Figure 23: Graph showing binding of ERK2 (thrombin cleaved) to synthesised peptide having the amino acid sequence RSKAKNPLYR compared to the 15 mer RSKAKWQTGTNPLYR fragment of the cytoplasmic domain of the β6 subunit.

Figure 24: Graph showing binding of JNK-1 to the β6 cytoplasmic domain.

Figure 25: location of β6 Δ746-764, β6(770t) and β6(777t) deletions in the cytoplasmic domain of the β6 subunit.

Figure 26: SW480 cells transfected with wild-type full length coding sequence for β6 or β6 Δ746-764 deletion mutant analysed by FACScan for expression of wild-type or mutant β6. White and black histograms represent cells stained in the absence and presence of the integrin subunit, respectively.

Figures 27(A) and 27(B): (A) Western blotting: equal protein loads of cell lyates from SW480 cells expressing wild-type β6 or β6 Δ746-764 deletion mutant. (B) β6 immunoprecipitates (mAb R6G9) from equal protein loads of cell lysates (A) probed with anti-ERK mAb (E10).

Figure 28: Proliferation of HT29 colon cancer cells cultured for 48 hours and treated with penetratin, the fragment of β6 cytoplasmic domain having amino acid sequence RSKAKWQTGTNPLYR alone or the fragment coupled to penetratin for the final 24 hours of the incubation period.

Figure 29: Proliferation of SW480 cells expressing wild-type β6 cultured on plastic for 48 hours and treated with penetratin, the RSKAKWQTGTNPLYR fragment alone or the fragment coupled to penetratin for the final 24 hours of the incubation period.

Expression of β6 is also up-regulated in migrating keratinocytes at the wound edge during experimental epidermal wound healing. αvβ6 is not expressed in normal epithelium (Jones et al, 1997). However, following experimental wounding, αv appears to switch its heterodimeric association from β5 to β6 subunit during re-epithelialisation. At day 3 after wounding, β6 is absent but then appears around the perimeter of the basal cells of the migrating epidermis (Clark et al, 1996). By day 14 after wounding, when re-epithelialisation is complete, all suprabasalar cells overlying the wound express β6 but not β5. In human mucosal wounds, maximal expression of β6 has been observed relatively late when epithelial sheets are fused and granulation tissue is present (Haapasalmi et al, 1996). Furthermore, those investigators observed maximal expression of tenascin with αvβ6 expression. Interestingly, freshly isolated keratinocytes have not been found to express β6 but begin to express this after subculturing (Haapasalmi et al, 1996). Moreover, TGF-β1 has been shown to induce the *de novo* expression of αvβ6 at the cell surface on keratinocytes (Zambruno et al, 1995). This is particularly relevant in view of the recent observation that αvβ6 binds and activates latent TGF-β1 which may be a means of locally regulating TGF-β1 function *in vivo* during tissue response to injury (Munger et al, 1999).

αvβ6 expression is also upregulated in type II alveolar epithelial cells during lung injury caused by injection of live bacteria and αvβ6 mRNA is induced within 5 hours of acute injury (Breuss et al, 1995). Interestingly, αvβ6 has been shown to be expressed on proximal airway epithelial cells in 50% of smokers undergoing lung resection (Weinacker et al, 1995). Just as in human keratinocytes, in primary cultures of human airway epithelial cells, TGF-β1 has been shown to dramatically increase expression of

extracellular matrix (Agrez, 1989), and the observation that induced expression of αVβ6 in colon cancer cells markedly enhances gelatinase B secretion (Agrez et al, 1999).

The β6 subunit is widely observed in cancers of various origins (Breuss et al, 1995). As described above, β6 is detected in at least 50% of bowel cancer tumours. Others have reported its presence in oropharyngeal cancers where it is also present and strongly expressed in the invading margins of the cancer cell islands as is commonly found in bowel cancer. In the oropharyngeal mucosa, no β6 is observed in the normal lining cells of the mouth but in both primary and metastatic tumours from the oropharyngeal mucosa, strong β6 expression is seen which does not correlate with degree of differentiation and in particular, is restricted to the basal layer of epithelial cells. In colon cancer, β6 expression is similarly maximal at the advancing edges of tumour cell islands (Agrez et al, 1996).

Hence, modulation of MAP kinase interaction with the β6 subunit in epithelial cells may be used in the prophylaxis or treatment of cancer of the lip, tongue, salivary glands, gums, floor and other areas of the mouth, oropharynx, nasopharynx, hypopharynx and other oral cavities, oesophagus, stomach, small intestine, duodenum, colon, rectum, gallbladder, pancreas, larynx, trachea, bronchus, lung, female and male breast, uterus, cervix, ovary, vagina, vulva, prostate, testes, penis, bladder, kidney, thyroid and skin.

In terms of prophylactic use, a method of the invention may find application in protecting against ultraviolet-induced skin cancer, lung cancer in smokers, cancer of the gut where polyps are present as in polyposis coli or other inheritable disease where a pre-disposition to the development of polyps exists, breast cancer in high risk patients with a

Rather than replacing a defective gene with one encoding a polypeptide the expression of which restores normal function of the cell as in the above examples, the possibility exists to achieve down regulation of cancer cells such as colon cells by introducing a gene that encodes an integrin subunit in which the binding domain for interacting with a MAP kinase has been rendered defective by mutagenesis, or in which the binding domain has been wholly or partially deleted, to thereby achieve down regulation through the inhibition of the MAP kinase integrin interaction. The defective integrin subunit will nevertheless usually be able to associate with its normal partner integrin subunit and be expressed on the cell membrane. Preferably, the defective integrin subunit will be expressed at a higher level than the corresponding wild-type integrin subunit such that down regulation is achieved by a dominant negative effect. Alternatively, such therapy may involve the introduction and expression of a nucleic acid sequence that encodes a fragment or truncated form of an integrin subunit that excludes the binding domain for the MAP kinase or in which the binding domain has been partially or wholly deleted or otherwise mutagenised so as to be defective.

Another option is to introduce a nucleic acid sequence encoding a polypeptide capable of binding to the binding domain on the integrin for the MAP kinase or to the binding site on the MAP kinase upon being expressed within the cell to thereby inhibit binding of the MAP kinase to the integrin and thereby achieve down regulation of cellular activity.

A gene or nucleic acid sequence encoding an integrin subunit may also be modified such that although the encoded binding domain for the MAP kinase remains unaltered, the amino acid sequence of a region of the integrin subunit distant from the

target nucleic acid sequence, or which first nucleic acid molecule is capable of being transcribed to a nucleic acid molecule capable of interacting with the target sequence whereby the interaction of the first nucleic acid molecule with the target sequence inhibits expression of the integrin subunit.

Reference to the first nucleic acid molecule is to be understood as a reference to any nucleic acid molecule which directly or indirectly facilitates reduction, inhibition or other form of down regulation of the expression of the integrin molecule. Nucleic acid molecules which fall within the scope of this definition include antisense sequences administered to a cell and antisense sequences generated *in situ* which have sufficient complementarity with target sequence such as mRNA encoding the integrin subunit or for instance, a transcription regulatory sequence controlling transcription of the gene encoding the integrin subunit, to thereby be capable of hybridising with the target sequence and inhibit the expression of the integrin subunit. The first nucleic acid molecule may also be a ribozyme capable for instance, specifically binding to that region of nucleic acid encoding the binding domain of an integrin subunit and cleaving the nucleic acid.

On *a priori* grounds, targeting the expression of the β6 subunit in malignant cells such as in colon cancer by means of adenoviral-mediated antisense therapy is preferred because down-regulating β6 by means of a non-adenoviral approach may increase cell surface expression of the β5 subunit. The relevance to such therapy is that the vitronectin-binding integrin αvβ5 promotes adenovirus internalisation (Thomas et al, 1993). Given that abundant αvβ5 is always present on the surface of colon cancer cells for example, a secondary benefit of inhibiting β6 expression during the course of therapy

endogenous exonucleases and or endonucleases to provide *in vivo* stability in target cells. Modification to the phosphate backbone, sugar moieties or nucleic acid bases may also be made to enhance uptake by cells or for instance solubility, and all such modifications are expressly encompassed. Such modifications include modification of the phosphodiester linkages between sugar moieties, the utilisation of synthetic nucleotides and substituted sugar moieties, linkage to liphophilic moieties and the such like as described in US Patent No. 5,877,309. Methods for the construction of oligonucleotides for use in antisense therapy have previously been described (see Van der Krol et al. 1998 Biotechniques 6:958-976; and Stein et al. 1998 Cancer Res 48:2659-2668; Bachman et al. 1998, J. Mol. Med. 76:126-132).

Any means able to achieve the introduction of a gene or a nucleic acid into a target cell may be used. Gene transfer methods known in the art include viral and non-viral transfer methods. Suitable virus into which appropriate viral expression vectors may be packaged for delivery to target cells include adenovirus (Berkmer, 1992; Gorziglia and Kapikian, 1992); vaccinia virus (Moss, 1992); retroviruses of avian (Petropoulos et al, 1992); murine (Miller, 1992) and human origin (Shimada et al, 1991); herpes viruses including Herpes Simplex Virus (HSV) and EBV (Margolskee, 1992; Johnson et al, 1992; Fink et al, 1992; Breakfield and Geller, 1997; Freese et al, 1990); papovaviruses such as SV40 (Madzak et al, 1992), adeno-associated virus (Muzyczka, 1992); BCG and poliovirus. Particularly preferred virus are replication deficient recombinant adenovirus (eg. He et al, 1998). Engineered virus may be administered locally or systemically to achieve delivery of the gene or nucleic acid sequence of interest into a target cell.

MAP kinase with the integrin, as well as allostearic inhibitors that distort the binding domain upon associating with the integrin.

The binding domain of an integrin may be identified and characterised using protocols and techniques described herein. Specifically, a binding domain may be localised by assessing the capacity of respective overlapping peptide fragments corresponding to different regions of the cytoplasmic domain of an integrin subunit to associate with a MAP kinase. The specific amino acid sequence which constitute the binding domain for the MAP kinase may then be determined utilising progressively smaller peptide fragments of the region of the cytoplasmic domain of the integrin subunit observed to interact with the MAP kinase. In particular, test peptides are readily synthesised to a desired length involving deletion of an amino acid or amino acids from either or both ends of the amino acid sequence corresponding to that region each time, and tested for their ability to associate with the MAP kinase. This process is repeated until the minimum length peptide capable of associating with the MAP kinase substantially without compromising the optimum observed level of association is identified. The specific amino acids that play an active role in the interaction with the MAP kinase is achieved with the use of further synthesised test peptides in which one or more amino acids of the sequence are deleted or substituted with a different amino acid or amino acids to determine the effect on the ability of the peptide of associate with the MAP kinase. Typically, substitution mutagenesis will involve substitution of selected ones of the amino acid sequence with alanine or other relatively neutrally charged amino acid. By deletion is meant deletion of one or more of the amino acids between the N-terminal and C-terminal amino acid residues of the identified amino acid sequence.

The design of a mimetic of the binding domain will usually involve selecting or deriving a template molecule onto which chemical groups are grafted to provide required physical and chemical characteristics. The selection of template molecule and chemical groups is based on ease of synthesis, likely pharmacological acceptability, risk of or potential for degradation *in vivo*, stability and maintenance of biological activity upon administration. Pharmacological acceptability and the like are also taken into consideration in the design of other agent types.

In order to constrain a polypeptide or other agent in a three dimensional conformation required for binding, it may be synthesised with side chain structures or otherwise be incorporated into a molecule with a known stable structure *in vivo*. In particular, a polypeptide or the like may be incorporated into an amino acid sequence at least part of which folds into a β-pleated sheet or helical structure such as an α-helix (eg. see Dedhar et al., 1997).

A polypeptide or other agent may also be cyclised to provide enhanced rigidity and thereby stability *in vivo*. Various methods for cyclising peptides, fusion proteins or the like are known (eg. Schiller et al., 1985). For example, a synthetic peptide incorporating two cysteine residues distanced from each other along the peptide may be cyclised by the oxidation of the thiol groups of the residues to form a disulfide bridge between them. Cyclisation may also be achieved by the formation of a peptide bond between the N-terminal and C-terminal amino acids of a synthetic peptide or for instance through the formation of a bond between the positively charged amino group on the side chain of a lysine residue and the negatively charged carboxyl group on the side chain of a glutamine acid residue. As will be understood, the position of the various amino acid

observed functional activity in the presence of the test agent is indicative of the modulating effect provided by the test agent.

It will also be understood that the integrin in the context of such assays may be an integrin subunit or polypeptide or fragment incorporating the binding domain of the integrin for the MAP kinase, or a homolog, analog, variant or derivative of such a molecule to which the MAP kinase is capable of binding. In addition, determination of whether an agent is capable of binding to the binding domain of an integrin may be readily achieved by using a polypeptide or fragment as described herein consisting of the binding domain of the integrin or core amino acid sequence of the binding domain that directly participates in the binding interaction with the MAP kinase or analogs or the like of such molecules.

It is not necessary that an agent be proteinaceous in character and indeed, mimetics may be prepared which may not be a polypeptide at all but which nevertheless possess the capability of binding with the integrin.

Polypeptides including fusion proteins and fragments of an integrin subunit comprising the binding domain for a MAP kinase or incorporating sufficient core amino acid sequence of the binding domain for binding by the MAP kinase are encompassed by the present invention. Typically, a polypeptide of the invention will have a length of about 150 amino acids or less, more preferably about 100 or 50 amino acids or less and generally, less than about 40 amino acids. Preferably, the length will be from between about 5 to about 30 amino acids, and more preferably from between about 5 amino acids and about 25 amino acids. Preferably, a polypeptide will comprise or incorporate the

fluorophores, chemiluminescent agents and enzymes (see eg. Essential Molecular Biology, A Practical Approach Vol. II, Oxford University Press, 1993; Current Protocols in Molecular Biology, Ausubel FM., John Wiley & Sons Inc., 1998). The choice of a label will vary depending on the degree of sensitivity required, ease of conjugation with the probe, safety and other factors.

Oligonucleotides for use as probes or primers will usually have a length of less than about 60 nucleotides, usually less than about 50 or 40 nucleotides preferably, between about 14 and about 30 nucleotides, and more preferably, between about 14 and about 25 nucleotides. While it is desirable that a primer or probe has 100% complementarity with its target sequence, oligonucleotides may be designed with less complementarity but which nevertheless hybridise with the target sequence. Typically, a primer or probe will have a complementarity of about 70% or greater, more preferably about 80% or greater and most preferably about 90% or 95%, or greater. A probe will generally be designed for being capable of hybridising with its target nucleic acid sequence under moderate or high stringency wash conditions. Moderate stringency wash conditions are for example those that employ 0.2 x SSC (0.015M NaCl/0.0015M sodium citrate) /0.1% SDS (sodium dodecylsulfate) wash buffer at 42°C. High stringency wash conditions employ for instance, 0.1 x SSC wash buffer at 68°C. Generally, the content of purine relative to the content of pyrimidine nucleotides in the region of target nucleic acid of interest will be taken into account in the design of such primers and probes as will be their length in accordance with well accepted principles known in the art.

In addition, the present invention provides vectors incorporating nucleic acid sequences of the invention. The term "vector" is to be taken to mean a nucleic acid

may be utilised examples of which include pVL based vectors such as pVL1392, and pVL941, and pAcUW based vectors such as pAcUW1. Viral expression vectors are particularly preferred.

Typical cloning vectors incorporate an origin of replication (*ori*) for permitting efficient replication of the vector, a reporter or marker gene for enabling selection of host cells transformed with the vector, and restriction enzyme cleavage sites for facilitating the insertion and subsequent excision of the nucleic acid sequence of interest. Preferably, the cloning vector has a polylinker sequence incorporating an array of restriction sites. The marker gene may be drug-resistance gene (eg. Amp^r for ampicillin resistance), a gene encoding an enzyme such as chloramphenicol acetyltransferase (CAT), β-lactamase, adenosine deaminase (ADA), aminoglycoside phosphotransferase (APH), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), or for instance β-galactosidase encoded by the *E. coli lacZ* gene (*LacZ'*). Yeast reporter genes include imidazole glycerolphosphate dehydratase (HIS3), *N*-(5'-phosphoribosyl)-anthranilate isomerase (TRP1) and β-isopropylmalate dehydrogenase (LEU2). As will be appreciated, expression vectors of the invention may also incorporate such marker genes.

Cloning vectors include cloning vectors for mammalian, yeast and insect cells. Particular vectors that may find application include pBR322 based vectors and pUC vectors such as pUC118 and pUC119. Suitable expression and cloning vectors are for instance described in Molecular Cloning. A Laboratory Manual., Sambrook et al., 2nd Ed. Cold Spring Harbour Laboratory., 1989.

are known in the art include penetratins and variants thereof (Derossi et al. 1994, 1996), human immunodeficiency virus Tat derived peptide (Prochiantz, 1996), and transportan derived peptide (Pooga et al. 1998). Indeed, carrier peptides have been successfully used to facilitate internalisation of mimetics of Src homology 2 binding sites, and peptides which inhibit protein kinase C mediated axon development and CD44 (hyaluronate receptor) dependent migration (Theodore et al. 1995; Williams et al. 1997; Peck Isacke, 1998; Derossi et al. 1998).

Specific targetting to β6-expressing cancer cells may also be achieved by coupling humanised anti-β6 antibody to carrier molecules such as penetratin coupled to an agent capable of inhibiting binding of a MAP kinase with an integrin expressed by the cell or down regulation of the expression of the integrin. Coupling may for instance be by a peptide bond or disulfide bridge. Given that β6 expression enhances effective proteolysis at the cell surface by matrix metalloproteinase-9 (Agrez et al. 1999), such targetting approaches may include engineering an MMP-9 cleavage site between the antibody and the carrier peptide penetratin to facilitate internalisation of the pentratin-agent complex. Another approach may employ coupling the penetratin-agent complex to β6 integrin receptor-targetted peptides, targetted for binding to the extracellular β6 domain by virtue of their DLXXL sequence. For example, a ligand recognition motif for αVβ6 integrin, RTDLDSLRTYTL (Kraft et al. 1999) may be used in conjunction with or without an engineered MMP-9 cleavage site to release the penetratin-agent complex at the cell surface. Further protocol for targetting nucleic acids to cells by targetting integrins is described in Bachmann et al. 1998.

the like), vegetable oils, and suitable mixtures thereof. Fluidity may be maintained by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants.

Sterile injectable solutions will typically be prepared by incorporating the active agents in the desired amount in the appropriate solvent with various other components enumerated above, prior to sterilising the solution by filtration. Generally, dispersions will be prepared by incorporating the sterile active agents into a sterile vehicle which contains the dispersion medium and other components. In the case of sterile powders for the preparation of sterile injectable solutions, preferred methods of preparation are vacuum drying and freeze-drying techniques which yield a powder of the active agent plus any additional desired ingredient from previously sterile filtered solutions thereof.

For oral administration, the active agents may be formulated into any orally acceptable carrier deemed suitable. In particular, the active ingredient may be formulated with an inert diluent, an assimilable edible carrier or it may be enclosed in a hard or soft shell gelatin capsule. Alternatively, it may be incorporated directly into food. Moreover, an active agent may be incorporated with excipients and used in the form of ingestable tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, and the like.

Such compositions will generally contain at least about 1% by weight of the active agent or agents. The percentage may of course be varied and may conveniently be between about 5 to about 80% w/w of the composition or preparation. As will be appreciated, the amount of active agent or agents in such compositions will be such that a suitable effective dosage will be delivered to the subject taking into account the

Pharmaceutically acceptable carriers, diluents and or excipients include any suitable conventionally known solvents, dispersion media and isotonic preparations or solutions. Use of such ingredients and media for pharmaceutically active substances is well known. Except insofar as any conventional media or agent is incompatible with the active agent, use thereof in therapeutic and prophylactic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions if desired.

It is particularly preferred to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein is to be taken to mean physically discrete units suited as unitary dosages for the subject to be treated, each unit containing a predetermined quantity of active agent calculated to produce the desired therapeutic or prophylactic effect in association with the relevant carrier, diluent and/or excipient.

A unit dosage formed will generally contain each active agent in amounts ranging from about 0.5 μg to about 2000mg/ml of carrier respectively.

A pharmaceutical composition may also comprise vectors capable of transfecting target cells where the vector carries a nucleic acid molecule for modulating functional activity or expression of an integrin or MAP kinase. The vector may for instance, be packaged into a suitable virus for delivery of the vector into target cells as described above.

The dosage of an active agent will depend on a number of factors including whether the agent is to be administered for prophylactic or therapeutic use, the condition for which the agent is intended to be administered, the severity of the condition, the age

periodically challenged with the relevant antigen to establish and/or maintain high antibody titre. To produce monoclonal antibodies, B lymphocytes can be isolated from the immunised mammal and fused with immortalising cells (eg. myeloma cells) by standard somatic cell fusion techniques (eg. utilising polyethylene glycol) to produce hybridoma cells (Kohler and Milstein, 1975; see also Handbook of Experimental Immunology, Weir et al Eds. Blackwell Scientific Publications, 4th Ed. 1986). The resulting hybridoma cells may then be screened for production of antibodies specific for the peptide by an enzyme linked immunosorbant assay (ELISA) or other immunoassay. Conventionally used methods for preparing monoclonal antibodies include those involving the use of Epstein-Barr virus (Cole et al. Monoclonal Antibodies and Cancer Therapy, Allen R. Liss Inc. pp. 77-96, 1985). The term "antibody" or "antibodies" as used herein is to be taken to include within its scope entire intact antibodies as well as binding fragments thereof such as Fab and $(Fab')_2$ fragments which may be obtained by papain or pepsin proteolytic cleavage, respectively.

An antibody of the invention may be labelled for enabling detection of antibody binding in immunoassays including competitive inhibition assays. A "label" may be any molecule which by its nature is capable of providing or causing the production of an analytically identifiable signal which allows the detection of an antibody and antigen complex. Such detection may be qualitative or quantitative. An antibody can for instance be labelled with radioisotopes including ^{32}P, ^{125}I or ^{131}I, an enzyme, a fluorescent label, chemiluminescent molecule or for instance an affinity label such as biotin, avidin, streptavidin and the like.

specific for the antigen are also well known. An antibody can be bound to a solid substrate covalently utilising commonly used amide or ester linkers, or by adsorption. Optimal concentrations of antibodies, temperatures, incubation times and other assay conditions can be determined by the skilled addressee with reference to conventional assay methodology and the application of routine experimentation.

Antibodies and other molecules of the invention including polypeptides and oligonucleotides as described herein when bound to a solid support can be used in affinity chromatography for the purification of a binding partner for which they are specific. In particular, a polypeptide either alone or as a fusion protein comprising the binding domain for a MAP kinase for instance can be utilised in the purification, isolation or concentration of the MAP kinase. It may also be used for assaying levels of MMP kinase in cell extracts. Similarly, an antibody that specifically binds to the binding domain of an integrin has use in the purification of the integrin or fragments thereof incorporating the binding domain from a relatively crude preparation or mixture. Suitable solid supports include agarose, sepharose and other commercially available supports such as beads formed from latex, polystyrene, polypropylene, dextran, glass or synthetic resins, typically packed in an affinity column through which the relevant sample containing the binding partner is passed at a pH and conditions (eg., low salt concentration) under which the binding partner becomes bound by the antibody, polypeptide or other such molecule. The column is then washed utilising a suitable buffer whereby the binding partner is retained bound on the column, prior to being eluted therefrom utilising a suitable elution buffer (eg., with a higher salt concentration and at an altered pH, typically pH 2.5 or pH 11) that facilitates the release of the binding

inhibit tumour cell invasion *in vitro* and tumour metastases from melanoma in an animal model (Humphries et al. 1986; Gehlsen et al. 1988).

In colon cancer, the contribution made by the α5β1 receptor to regulation of growth appears to be ligand (fibronectin)-dependent. For example, induced expression of α5β1 in human colon cancer cells constitutively lacking this integrin has been shown to result in decreased tumour cell proliferation *in vitro* (Varner et al, 1995). Interestingly, when the appropriate ligand was present, cell proliferation was restored, indicating that the unoccupied receptor mediated a negative growth signal in these cells (Varner et al, 1995). Moreover, induction of α5β1 expression was associated with a marked reduction of tumourigenicity in immune-deficient mice. Failure to ligate all of the tumour cell α5β1 molecules with sufficient murine fibronectin most likely accounts for the *in vivo* tumour suppression in these studies (Varner et al, 1995). In the present study the effect of down-regulating αvβ6 expression on colon cancer growth was examined.

1.1 Methods

1.1.1 Generation of sense and antisense β6 constructs in pEF.PGK.puro vector.

For β6 antisense constructs, β6 cDNA was excised from the vector pcDNA1neo β6 (Weinacker et al, 1994) using the restriction enzymes SnaB1 and Xba1. This produced a 5' overhang (Xba1) and a 3' blunt end (SnaB1). The 5' overhang was blunted with Klenow (Promega) prior to ligation. pEF.PGK.puro vector (a gift from D. Huang, the Walter & Eliza Hall Institute, Melbourne Australia) was cut with EcoRV which produced blunt ends. The pEF.puro vector was de-phosphorylated using calf intestinal alkaline phosphatase (CIAP, Promega) and the β6 cDNA ligated overnight into

curves performed using a range of concentrations of puromycin established that WiDr and HT29 cells could be stably transfected with puromycin concentrations of 1.0µg and 2.5µg ml, respectively.

1.1.3 Assessment of β6 expression in the transfected cell lines

β6 expression was assessed by means of FACScan analyses of parent cell lines and clones generated therefrom by means of limiting-dilution experiments. Stability of the transfectants was confirmed regularly by repeated FACScan analyses, and surface biotinylation and immunoprecipitation as described below.

1.1.4 FACScan analyses

Monolayer cultures of cell lines were harvested with trypsin/EDTA and then blocked with goat serum at 4°C for 10 min. Cells were washed once with PBS, incubated with primary antibody against integrin subunits for 20 min at 4°C and then washed twice with PBS. Cells were then stained with secondary antibody conjugated with phycoerythrin for 20 min at 4°C, washed twice with PBS and resuspended in 0.5ml PBS prior to FACScan analysis (Becton Dickinson, Rutherford, New Jersey, USA).

1.1.5 Integrin immunoprecipitation

Cells were harvested with trypsin/EDTA, the trypsin neutralised with standard culture medium, and the cell pellets washed once with cold PBS. Cell pellets were then exposed to biotin-CNHS-ester (Sigma) in biotinylation buffer (10mM sodium borate, 150mM sodium chloride, pH 8.8) for 30 mins at 4°C with continuous slow mixing. Cell pellets were then centrifuged at 4°C, washed twice with cold PBS and exposed to lysis buffer (containing 100mM Tris, 150mM NaCl, 1mM $CaCl_2$, 1% Triton, 0.1% SDS and 0.1% NP-40 at pH 7.4 and containing 1mM phenylmethylsulfonyl fluoride (PMSF)) for

cycles of amplification were performed under the following conditions: 94°C 1 min, 54°C 1 min and 72°C for 1 min. The reaction was stopped by incubating at 72°C for 10 min. To verify that equal amounts of RT product from cells were subjected to PCR amplification, the same amounts of cDNA were amplified for the "house-keeping" gene GAPDH using specific primers. The same reaction conditions were used except that the annealing temperature was changed to 48°C and PCR amplification performed for 35 cycles.

1.2 Results

1.2.1 αvβ6 expression in HT29 and WiDr transfected cell lines

Transfection of the colon cancer cell lines HT29 and WiDr with the β6 gene construct in an antisense orientation resulted in a marked reduction of β6 expression at the transcript level and on the cell surface as shown in Figs 1 to 4. Transfection of cells with β6 in the sense orientation did not enhance β6 surface expression. However, a consequence of down-regulation of the β6 subunit in antisense transfectants was a marked increase in surface expression of the β5 subunit. The changes in surface expression of β6 and β5 subunits noted on FACScan analyses of antisense β6 transfectants was confirmed by surface-labelling cells with biotin and immunoprecipitating integrin subunits with either anti-β6 mAb (R6G9) or anti-β5 mAb (P1F6).

1.2.2 Effect of Suppression of αvβ6 expression on cell binding to fibronectin

The major substrate for αvβ6 is fibronectin and to investigate the effect that reduction in β6 surface expression in antisense β6 transfectants might have on cell-matrix adhesion, WiDr and HT29 antisense β6 transfectants were seeded on fibronectin

1.2.3 Effect of suppression of αvβ6 expression on tumour cell proliferation and tumour growth *in vivo*

To investigate the effect of diminished αvβ6 surface expression on cell proliferation *in vitro*, WiDr and HT29 antisense β6 transfectants were seeded as monolayers in 96-well microtitre culture plates (5,000 viable cells per well) in standard culture medium containing the puromycin selection antibiotic. Cells were pulsed with 1μCi (^{3}H)-thymidine (Amersham) per well for the last 24 hours of each experiment before automated harvesting and measurement of radioactivity. WiDr wild-type, mock and antisense β6 transfectants, and HT29 wild-type, mock, sense β6 and antisense β6 transfectants were harvested second daily during a six-day culture period.

A marked increase in thymidine incorporation was observed for WiDR and HT29 cells expressing normal levels of αvβ6 compared with antisense β6 transfectants (see Figs. 5 and 6).

1.2.4 Effect of Suppression of αvβ6 expression on Tumour Formation

The ability of HT29 antisense β6 transfactants to form tumours in immune-deficient mice was assessed.

BALB/C female athymic mice (8 weeks of age purchased from the Animal Resource Centre, Perth, Western Australia) were maintained under pathogen-free conditions and fed standard mouse chow and water *ad lib*. The mice were divided into groups of ten each and all mice within each group inoculated with a single cell line. Cells used were WiDr mock (transfected with vector alone) and antisense β6 transfected clones (clones 1 - 3) and HT29 mock, sense and antisense β6 cell lines. Mice received subcutaneous flank injections of 10^7 viable tumour cells suspended in 0.2ml of standard

1.3 Discussion of Results

Induced expression of β6 in Chinese hamster ovary (CHO) cells has been shown to result in decreased surface expression of the β5 integrin subunit which also partners αv (Weinacker et al, 1994). The concept of integrin switching depends on the availability of the promiscuous αv partner subunit. In the present study, the reverse was observed. As a consequence of down-regulation of β6 in colon cancer cells which constitutively express αvβ6, β5 surface expression increased, most likely secondary to increased availability of the αv subunit partner.

Heterologous expression of αvβ6 in colon cancer cells has previously been reported to enhance tumour growth in immune-deficient mice (Agrez et al, 1994). Suppression of αvβ6 expression in the present study was shown to result in nearly complete disappearance of tumours in 93% of animals following subcutaneous inoculation of tumour cells. Moreover, in the remaining 7% of animals, a 95% reduction in tumour size was observed over a six week period compared with large tumours seen in all animals injected with cells in which αvβ6 expression had not been perturbed. Similar findings have been described with loss of the classical vitronectin receptor αvβ3 in melanoma. For example, in experimental animal models, the loss of αvβ3 expression in melanoma cells has been shown to lead to reduced *in vivo* proliferation which is restored upon re-expression of the receptor (Felding-Habermann et al, 1992).

Although the mechanisms involved in αvβ6-mediated tumour growth remain to be elucidated, the present *in vitro* data show that loss of β6 expression is associated with decreased proliferative capacity of the cells. Taken together with the marked reduction in gelatinase B secretion seen for colon cancer cells transfected with antisense β6, the

PMSF, 4μg ml aprotonin, 2μg ml leupeptin and 1μg ml pepstatin, pH 7.4) and sonicated at a setting of 7, using a Soniprep 150 watt ultrasonic disintegrator for a total of 90 seconds in three 30 second pulses with an interval of 30 seconds between each pulse. Cellular debris was removed by centrifugation at 900g for 10 min at 4°C. The assay was performed on equal cell numbers using a MAP kinase assay system (Amersham Pharmacia Biotech, Uppsala Sweden). The ability of cells to transfer phosphate from [γ^{32} P]-ATP to a synthetic peptide that contains specifically a p42/p44 MAP kinase phosphorylation site was measured as described in the manufacturer's instructions. [^{32}P]-labelled peptides were spotted onto PE1-cellulose paper, unbound radioactivity was washed with 75mM phosphoric acid and bound [^{32} P]-labelled peptides were measured by liquid scintillation counting. Protein estimation was performed on each cell lysate used and enzyme activity calculated as described in the manufacturer's instructions. Where MEK inhibitors, PD98059 and U0126 were used, cells were cultured as described above and the inhibitors, at a final concentration of 40μM, were added one hour before the addition of serum to the medium.

2.1.2 Western blotting

To detect the MAP kinases ERK1/2, cells were lysed in lysis buffer containing 100mM Tris, 150mM NaCl, 1mM CaCl2, 1% Triton, 0.1% SDS and 0.5% NP-40 at pH7.4, supplemented with enzyme inhibitors (1mM PMSF, 1mM sodium orthovanadate, 1μg ml pepstatin A, 2.5μg ml aprotonin, 1mM benzamidine, 1μg ml leupeptin). Lysates were clarified by ultracentrifugation and equal protein loads electrophoresed in 10% SDS-PAGE under non-reducing conditions. Electrophoresed proteins were transferred to nitrocellulose membranes (Biotrace NT, Gelman Sciences, Ann Arbor, MI) in transfer

The integrin-depleted lysates were electrophoresed in 10% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membranes. Membranes were blocked with casein as for Western blotting and probed with anti-ERK monoclonal antibodies E10 and SC-1647. In parallel experiments, the sequentially immunoprecipitated β6 subunit bound to rabbit anti-mouse (RAM) coupled Sepharose B4 beads was also electrophoresed in 10% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membranes, and the membranes probed with anti-ERK monoclonal antibody E10 which recognises only phosphorylated forms of ERK1/2.

2.3 Results

2.3.1 Effect of serum on MAP kinase activity

The effect of MEK (MAP kinase kinase) inhibitors UO126 and PD98059 on MAP kinase activity in WiDr and HT29 wild-type cells was tested in the absence/presence of serum. Adherent cell monolayers on plastic were grown for 24 hrs in standard culture medium, then washed three times in PBS followed by 16 hrs in culture under serum-free conditions. Serum was then added for 30 min and MAP kinase activity assessed before and after addition of serum. The addition of serum markedly stimulated MAP kinase activity for both cell lines and was inhibitable by both MEK inhibitors.

2.3.2 Effect of altered β6 expression on MAP kinase activity following serum stimulation.

In these experiments, WiDr transfectants (3 mock and 3 antisense β6 clones), HT29 transfectants (mock and antisense β6) and SW480 β6 transfectants (mock and sense β6) were serum-starved for 16 hrs followed by 30 mins exposure to serum. Increased expression of αvβ6 was associated with a marked increase in MAP kinase

2.3.4 Effect of altered β6 expression on total cellular ERK and β6-bound ERK.

Equal protein loads from one representative clone each from WiDr mock and antisense β6 transfectants were electrophoresed, transferred to nitrocellulose and blotted with anti-ERK mAb (SC-1647) as shown in Fig.11(A). β6 immuno-precipitates from equal protein loads of the WiDr mock and antisense β6 clones were electrophoresed, transferred and probed with anti-ERK mAb (E10) as shown in Fig. 11(B). As indicated, suppression of β6 expression resulted in a reduction of both total cellular and phosphorylated integrin-associated ERK compared with WiDr mock transfectants. Similarly, β6 immunoprecipitations from SW480 β6 transfected clones expressing high and low levels of β6 (confirmed by FACScan and β6 immunoprecipitations) showed parallel changes in β6-bound phosphorylated ERK (Fig. 12).

2.3.5 Effect of β6 immunodepletion on total cellular ERK and β6-bound ERK

WiDr wild-type cells were surface biotinylated and the cell lysates immunodepleted of β6 in three rounds of sequential immunoprecipitation using anti-β6 mAb (R6G9) resulting in a marked loss of β6 from the lysates as shown in Fig. 13(A). β6-immunodepleted lysates were then transferred and blotted with anti-ERK1/2 antibody (SC-1647), which recognises both phosphorylated and non-phosphorylated forms of ERK1/2. As shown in Fig. 13(B), following three rounds of β6 immunodepletion, levels of ERK1/2 in β6-depleted lysates compared with non-immunodepleted lysates, were markedly reduced suggesting a significant contribution of β6-bound ERK to total cellular ERK. In contrast, immunodepletion of β5 by three successive rounds of immunoprecipitation with mAb P1F6 or isotype-matched control antibody IgG2A did

leupeptin). 10 µl (10 µg of protein) was used for the analyses of cell lysates after adding equal volumes of non-reducing Laemmli buffer. For SW480-β6-transfected cells the rest of the cell lysate was used for immunoprecipitation of αvβ6 integrin. Cell lysates and β6 immunoprecipitates were subjected to western blotting and probed with E10 monoclonal antibody (recognising phosphorylated ERK1/2).

Cell lysates from the non-attached cells were found to require serum factors to maintain phosphorylation of total cellular ERK. In contrast, non-attached SW480 β6 colon cancer cells do not require serum factors to maintain the activation (phosphorylation) state of β6-bound ERK.

2.3.8 Effect of PP2A phosphatase on αvβ6-bound ERK

In experiments to examine the effect of protein phosphatase 2A (PP2A) on β6-bound ERK, SW480 β6-transfected cells were sonicated in buffer comprising 50mM Tric-HCl (pH 8.0), 10mM $MgCl_2$ and 0.01mM EGTA together with enzyme inhibitors, and cell lysates from both serum-starved and serum-induced cells treated with 0.5 units of PP2A (Promega) at 30°C for 10 minutes. The reaction mixture was stopped by addition of equal volumes of non-reducing Laemmli sample buffer. In parallel, PP2A-treated cell lysates were immunoprecipitated with mAb R6G9 (anti-β6) followed by Western blot with anti-ERK mAb E10. MAP kinase activity assays were performed according to the manufacturer's instructions (Amersham Pharmacia Biotec) using γ^{32}P-ATP.

Exposure of cell lysates prepared from serum-supplemented and serum-starved cells to the PP2A catalytic subunit resulted in dephosphorylation of total ERK. In contrast, dephosphorylation of β6-bound ERK was not observed in β6.

2.3.10 Inhibition of MAP kinase activity inhibits secretion of gelatinase B.

SW480 β6 transfectants were cultured under serum-free conditions for 48 hours in the absence presence of the MEK inhibitor PD98059 (40μM) or DMSO (vehicle control). Tumour-conditioned medium was assayed for gelatinase B by analysis of equal protein loads in a gelatin zymogram.

Inhibition of MAP kinase activity by the MEK inhibitor reduced gelatinase B secretion compared with controls.

2.3.11 β6-ERK2 association in HaCaT and HaRas Cell Lines

β6 immunoprecipitates were prepared from human kerotinocyte cell lines (HaCaT and HaRas were obtained from Prof. N. Fusenig, The German Cancer Research Institute, Heidelberg, Germany) using mAb R6G9 (anti-β6) and the immunoprecipitates probed with mab E10 (against phosphorylated ERK 1/2). Fig. 14 shows that ERK2 associates with β6 in both of HaCat and HaRas cells.

2.4 Discussion of Results

The MAP kinase pathway has been shown to be important in experimental tumour metastases (Mansour et al, 1994) and recent data implicate MAP kinases in tumour growth and invasiveness of colon cancer cells (Sebolt-Leopold et al, 1999). In the present study, up- down-regulation of β6 expression in various colon cancer cell lines was shown to enhance suppress respectively, MAP kinase activity. The presence of serum induced a three-fold increase in MAP kinase activity above that observed for serum-starved β6-expressing cells. In contrast, only a one-fold increase in serum-dependent MAP kinase activity was observed for cells in which β6 had been down-regulated consequent upon transfection with antisense β6. Moreover, induced

progression. Taken together with the finding that inhibition of MAP kinase activity by the MEK inhibitor PD98059 diminished gelatinase B secretion in β6-expressing cells, it seems that activation of MAP kinase signalling plays a role, at least in part, in β6-mediated induction of gelatinase B secretion.

Example 3

3.1 Identification of the binding domain on the β6 subunit cytoplasmic tail domain for ERK2

Peptide fragments corresponding to regions of the cytoplasmic tail domain of the β6 subunit were screened in an enzyme-linked immunosorbent assay (ELISA) for binding with ERK2. The 52 amino acid long β6 cytoplasmic tail is shown in Fig. 16 as are the amino acid sequences for the cytoplasmic domains of the β1 to β3 subunits. In particular, four synthetic peptides designated fragment 1 to fragment 4 were prepared and biotinylated at the N-terminal end of each, respectively (Auspep Pty Ltd, Melbourne Australia).

The region of the β6 tail to which each corresponds is indicated in Fig. 16 and set out below.

Fragment 1: HDRKEVAKFEAERSKAKWQTGT

Fragment 2: RSKAKWQTGTNPLYRGSTST

Fragment 3: NPLYRGSTSTFKNVTYKHRE

Fragment 4: FKNVTYKHREKQKVDLSTDS

The fragments overlap by 10 amino acids and are each 20 amino acids long with the exception of the fragment 1 with a length of 22 amino acids. Fragment 4 was

As shown in Fig. 17, significant binding of non-phosphorylated GST.ERK2 (0.25µg 100µl) to peptide fragment 2 was observed (1µg 100µl) while only negligible or low level binding for the other fragments was found.

Significant binding of non-phosphorylated ERK2 to both fragment 2 and β6 cytoplasmic tail peptide compared to fragments 1, 3 and 4 over a range of concentrations of ERK2 was also observed (see Fig.18). Similar results were observed using a range of concentrations of the peptide fragments as shown in Fig. 19.

To further localise the binding domain on the cytoplasmic tail of the β6 subunit, progressively shorter peptides from the region of the β6 cytoplasmic tail corresponding to peptide fragment 2 were synthesised, biotinylated and the capacity to associate or otherwise bind to ERK2 assessed as described above. The binding of GST.ERK2 to a 15 mer test peptide (seq. 4) having the amino acid sequence RSKAKWQTGTNPLYR and a 10 mer test peptide having the sequence RSKAKWQTGT is shown in Fig. 20 compared to fragment 2 over a range of concentrations of the peptides. As can be seen, no reduction in binding to the seq. 4 peptide compared to fragment 2 was found. Binding of ERK2 to the seq. 3 peptide was substantially less than that observed for seq. 4.

A number of 10 mer biotinylated peptides corresponding to regions of fragment 2 or fragment 3 were then tested. The amino acid sequence for each peptide is as follows and their location in the β6 cytoplasmic domain is indicated in Fig. 21.

10(1): NPLYRGSTST

10(2): WQTGTNPLYR

10(3): KEEAERSKAK

antibody. Absorbance was read at 405nm and the results are shown in Fig. 24. Significant binding of JNK-1 to the β6 cytoplasmic tail peptide was found.

Example 5

5.1 Evaluation of ability of ERK2 to bind to β6 Δ746-764 deletion mutant.

To examine the role of the amino acid sequence RSKAKWQTGTNPLYR in the β6 cytoplasmic domain *in situ*, a β6 deletion construct lacking the coding sequence for AERSKAKWQTGTNPLYRG was transfected into colon cancer cell line SW480 which does not constitutively express the αVβ6 integrin using the calcium phosphate method previously described for transfections into this cell line (Agrez et al, 1994). The location of the β6 Δ746-764 deletion is indicated in Fig. 25. Construction of the β6 Δ746-764 deletion mutant in the vector pcDNA1neo and failure of the expressed receptor to localise to focal adhesions in Chinese hamster ovary cells has been reported (Cone et al, 1994). Facscan analysis revealed comparable levels of surface expression of mutant β6 to that seen for the full length wild-type receptor (see Fig. 26).

Equal protein loads of cell lysates prepared from SW480 cells were immunoprecipitated with either anti-β6 monoclonal antibody (mAb R6G9) or matched isotype control antibody. Surface biotinylation prior to immunoprecipitation confirmed equal surface expression of mutant and wild-type β6 (see Fig. 27 (A). Aliquots of the immunoprecipitates were electrophoresed and transferred to nitrocellulose for Western blotting using monoclonal antibody E10 which recognises ERK1/2. As seen in Fig. 27(B), loss of the RSKAKWQTGTNPLYR sequence in the β6 cytoplasmic domain

In particular, at the termination of experiments, 30µl of WST-1 was added to 270µl culture medium volume in each microtitre well and the colour change quantitated in an ELISA plate reader by measuring absorbance of the formazan product at 450mn (using a reference wavelength of more than 600nm). The mean absorbance readings from triplicate wells (± standard error of the means) after subtraction of background control wells (culture medium without cells)was determined. Only the carrier penetratin-peptide complex was effective in inhibiting cell proliferation in contrast to either peptide or penetratin alone as shown in Figs. 28 and 29 indicating that the penetratin-peptide complex was internalised by both the HT29 and SW480 cells resulting in the observed suppression of colon cancer growth. Photographs of the SW480 cells treated with penetratin alone or the penetratin-peptide complex are shown in Fig. 30 (A) to (C).

Although the present invention has been described hereinbefore with reference to a number of preferred embodiments, the skilled addressee will understand that numerous variations and modifications are possible without departing from the scope of the invention.

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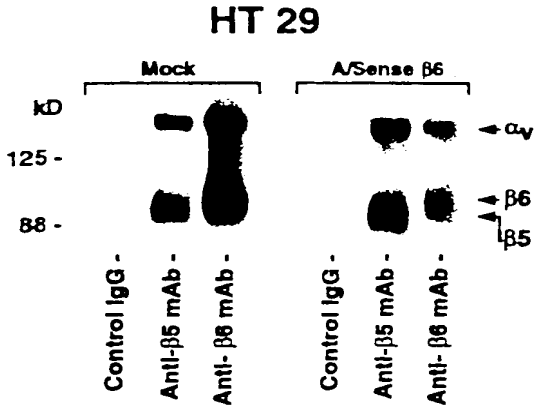

Figure 1

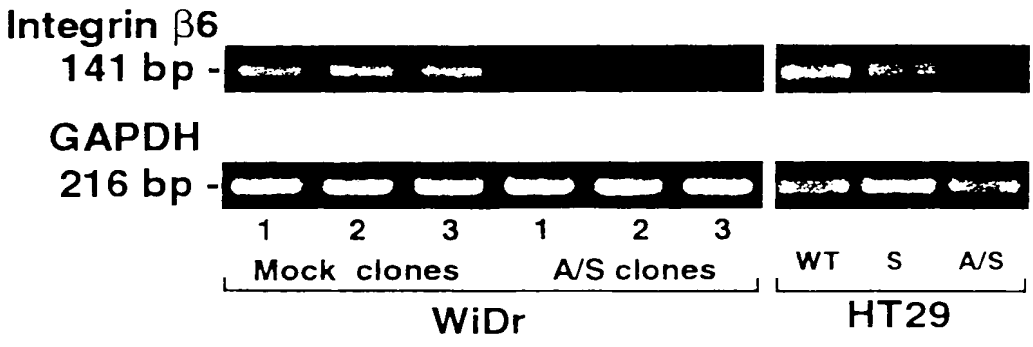

Figure 2

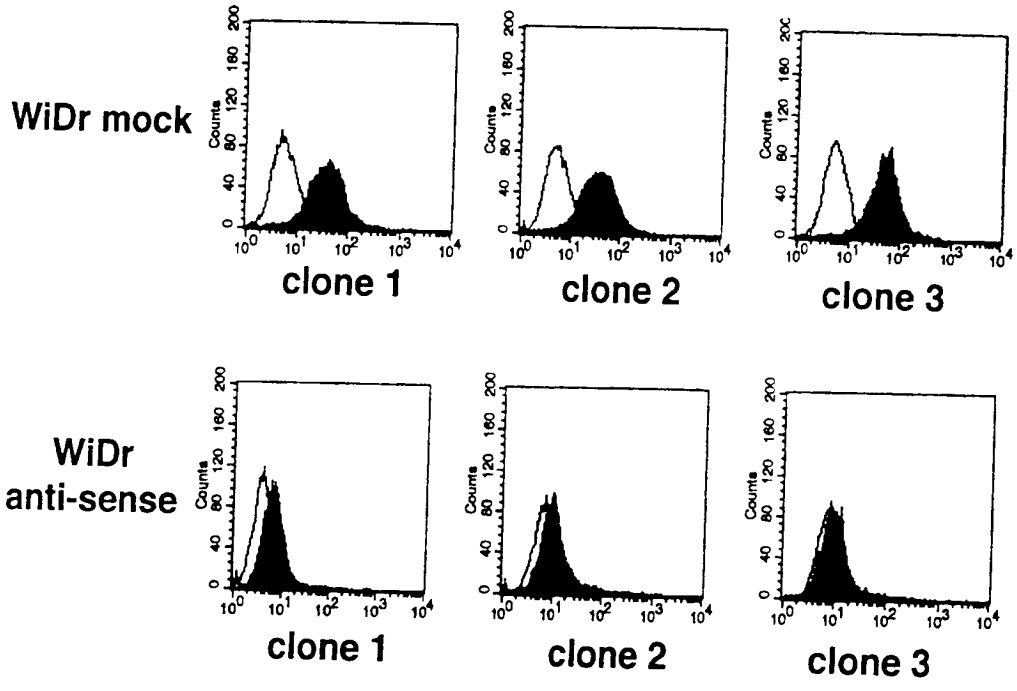
WiDr mock
clone 1
clone 2
clone 3
WiDr
anti-sense
clone 1
clone 2
clone 3
Counts

Figure 4

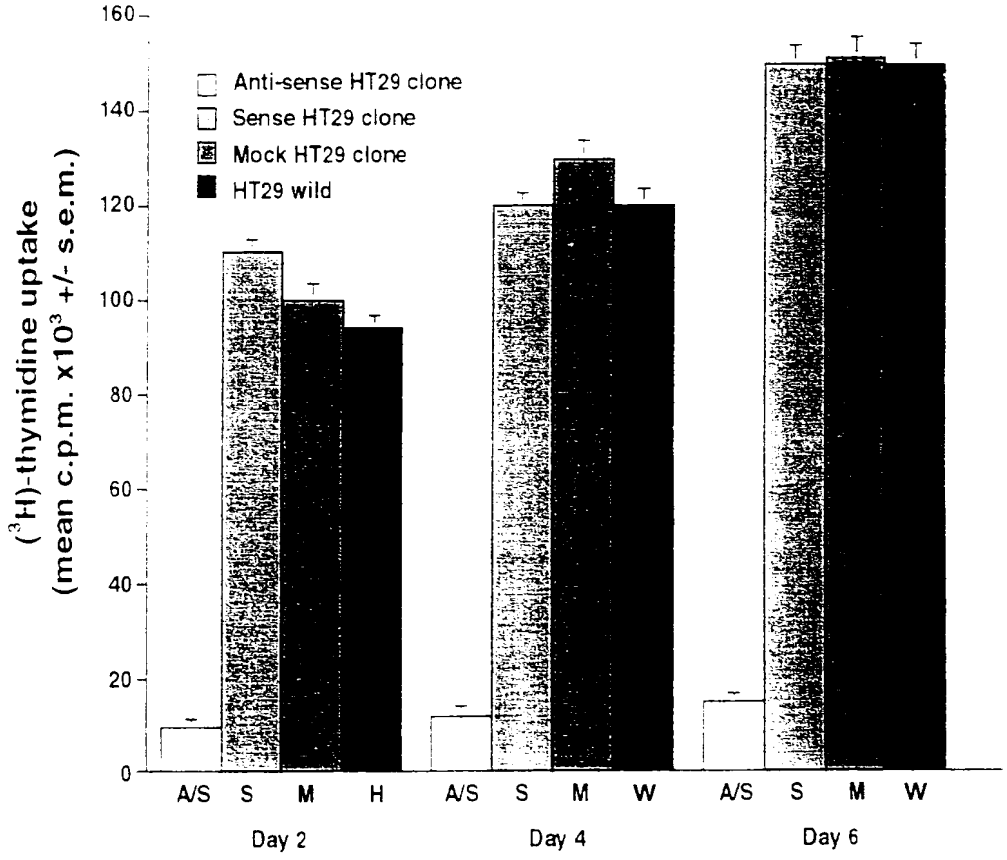
160
140
120
100
80
60
40
20
0
Anti-sense HT29 clone
Sense HT29 clone
Mock HT29 clone
HT29 wild
(^{3}H)-thymidine uptake
(mean c.p.m. x10^3 +/- s.e.m.)
A/S
S
M
H
A/S
S
M
W
A/S
S
M
W
Day 2
Day 4
Day 6

Figure 6

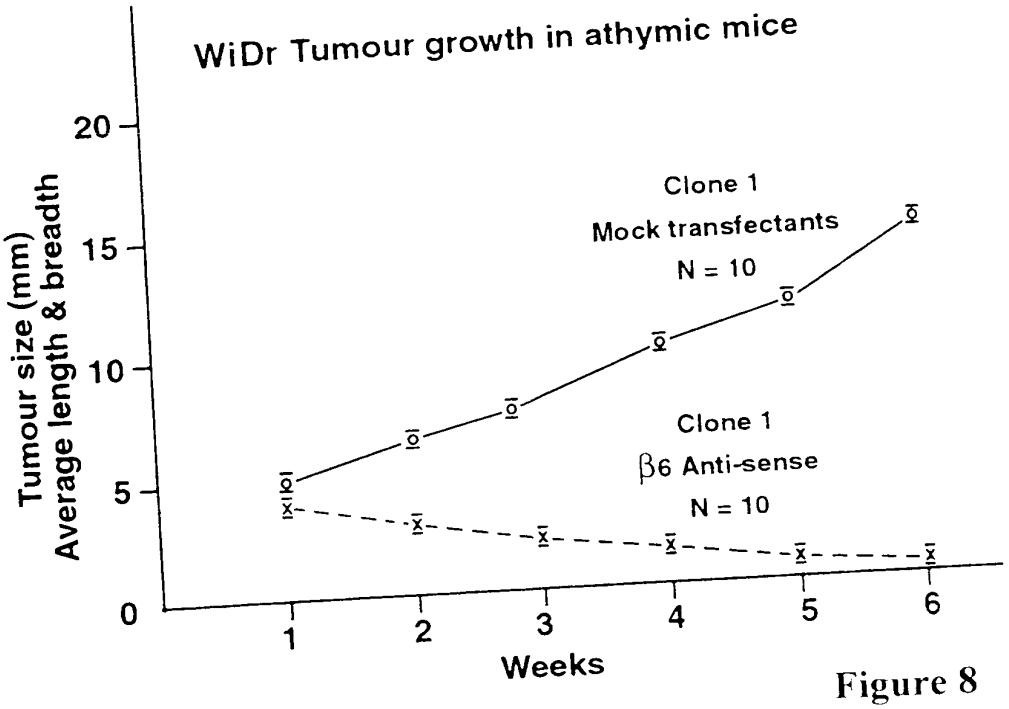

Figure 8

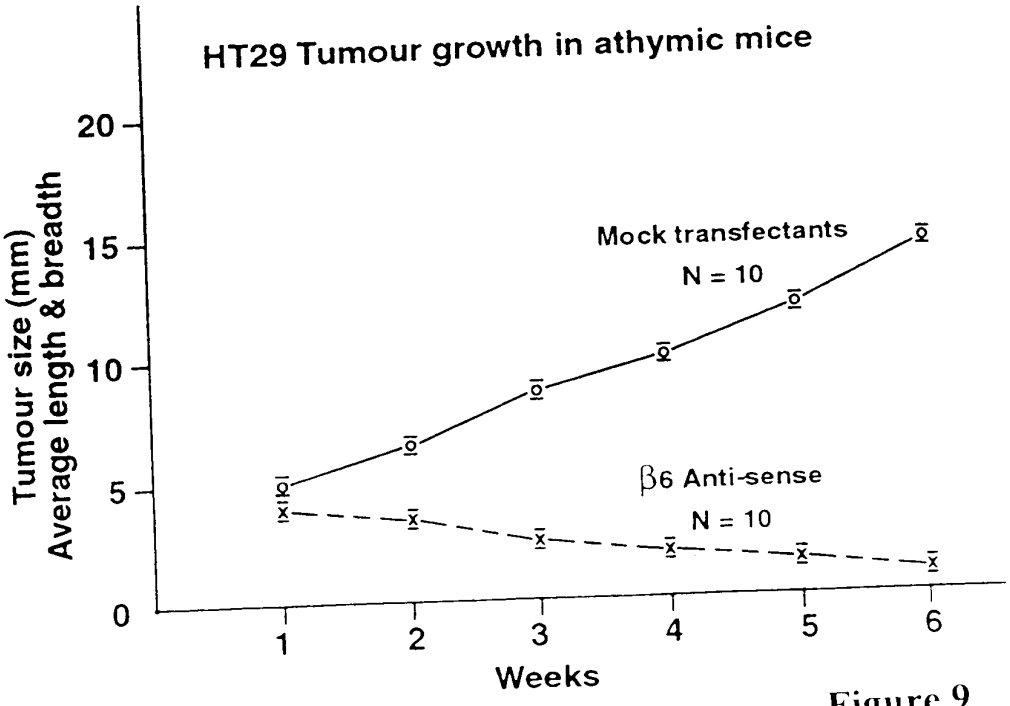

Figure 9

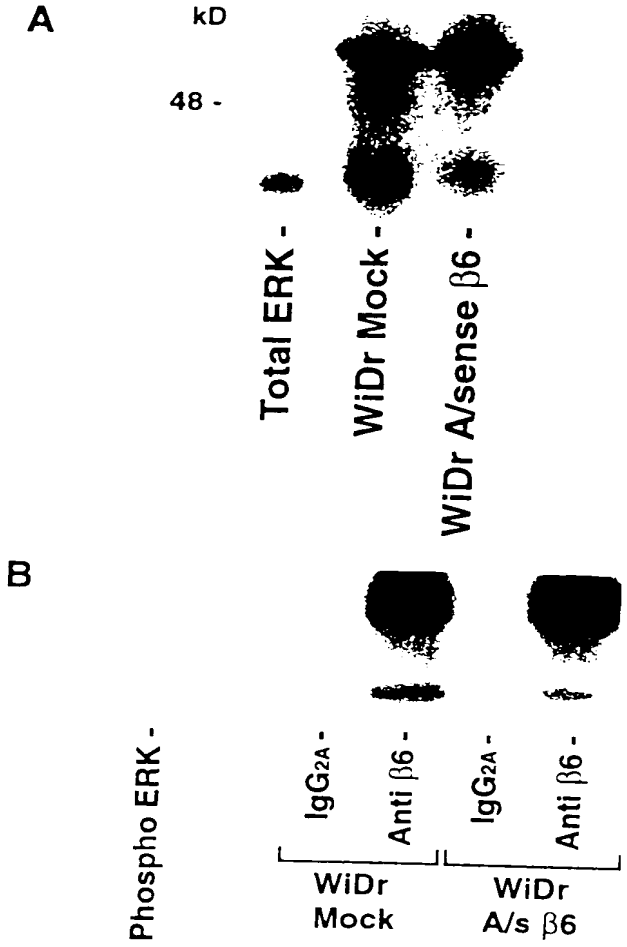
A
kD
48 -
Total ERK -
WiDr Mock -
WiDr A/sense β6 -
B
Phospho ERK -
IgG2A -
Anti β6 -
IgG2A -
Anti β6 -
WiDr Mock
WiDr A/s β6

Figure 11

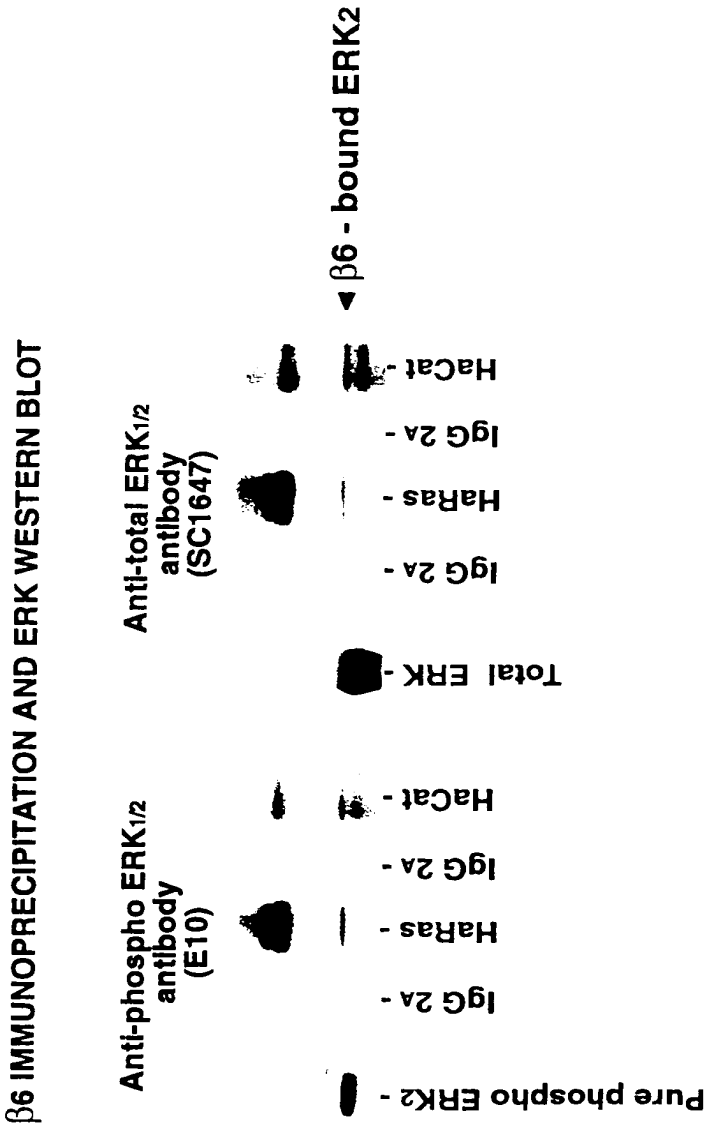
β6 IMMUNOPRECIPITATION AND ERK WESTERN BLOT
Anti-phospho ERK1/2 antibody (E10)
Anti-total ERK1/2 antibody (SC1647)
Pure phospho ERK2
IgG 2a
HaRas
IgG 2a
HaCat
Total ERK
IgG 2a
HaRas
IgG 2a
HaCat
β6 - bound ERK2

Figure 14

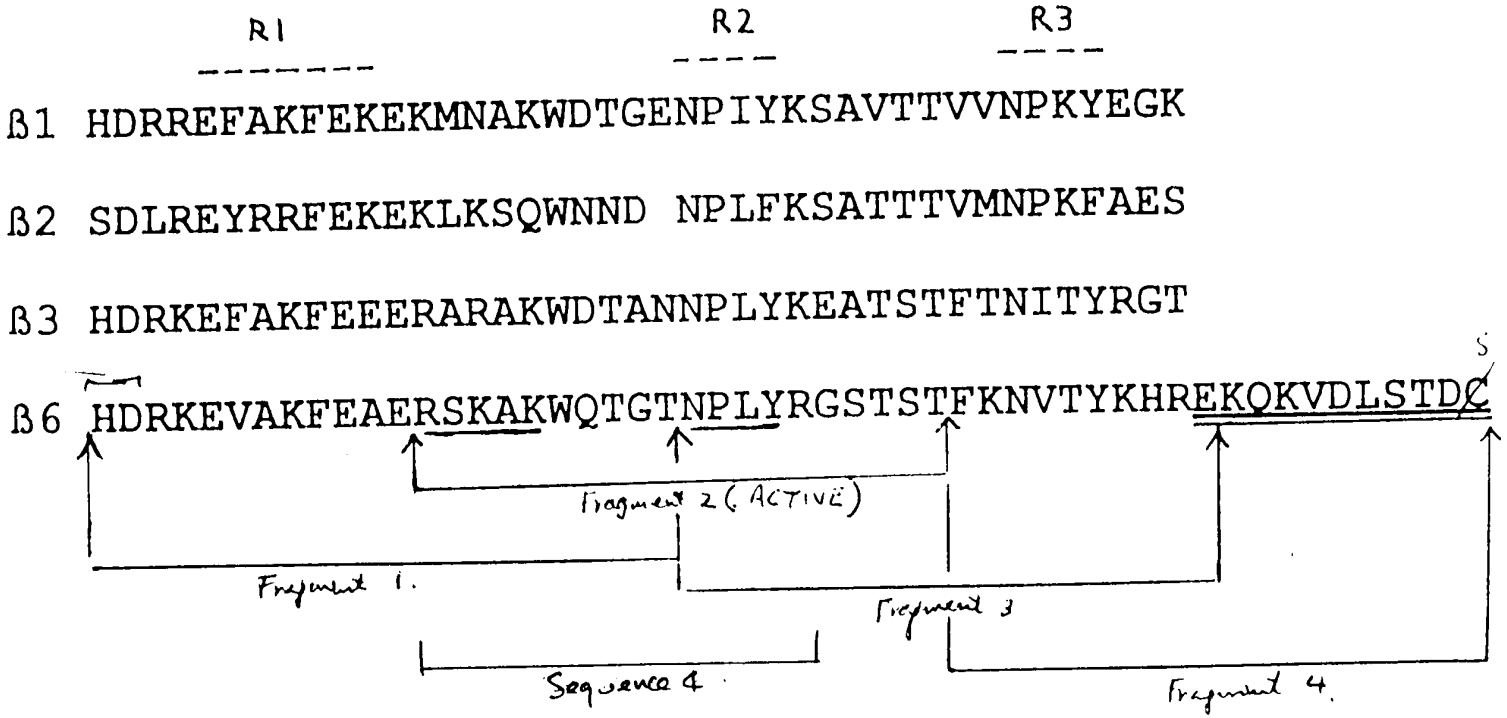
R1
R2
R3
ß1 HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK
ß2 SDLREYRRFEKEKLKSQWNND NPLFKSATTTVMNPKFAES
ß3 HDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT
ß6 HDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC
Fragment 2 (ACTIVE)
Fragment 1.
Fragment 3
Sequence 4
Fragment 4.

Figure 16

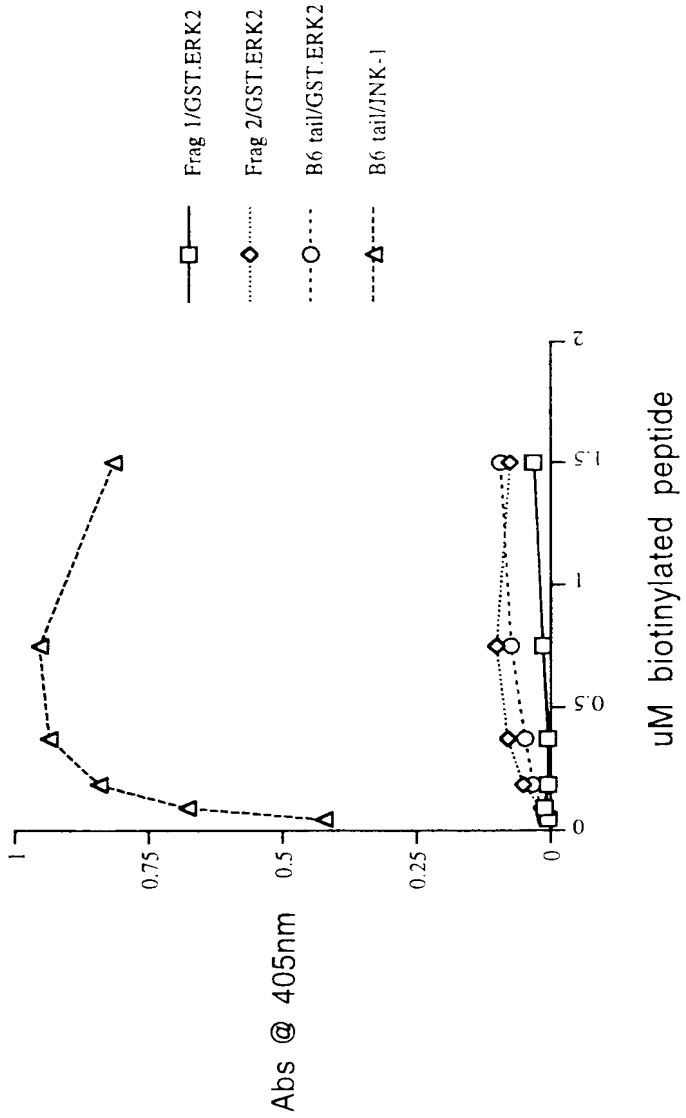
Frag 1/GST.ERK2
Frag 2/GST.ERK2
B6 tail/GST.ERK2
B6 tail/JNK-1
Abs @ 405nm
1
0.75
0.5
0.25
0
0
0.5
1
1.5
2
uM biotinylated peptide

Figure 19

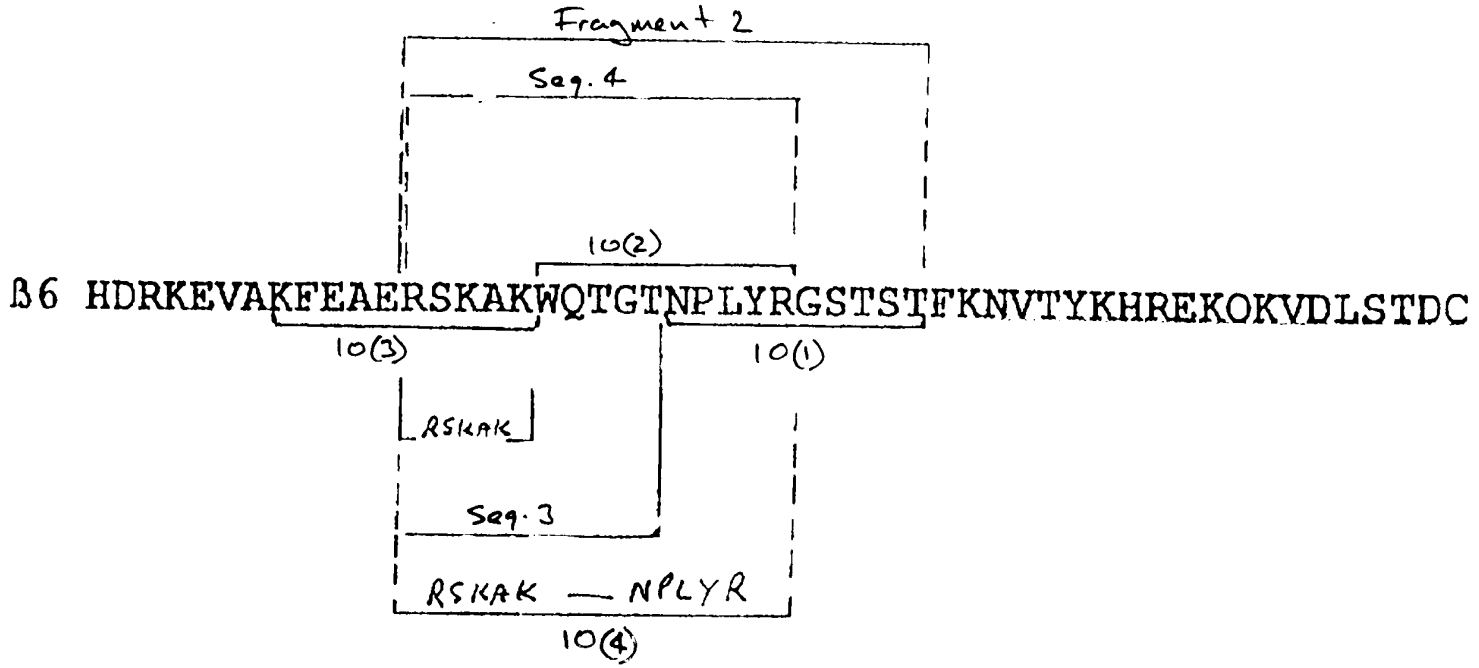
Fragment 2
Seq. 4
10(2)
ß6 HDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKOKVDLSTDC
10(3)
10(1)
RSKAK
Seq. 3
RSKAK — NPLYR
10(4)

Figure 21

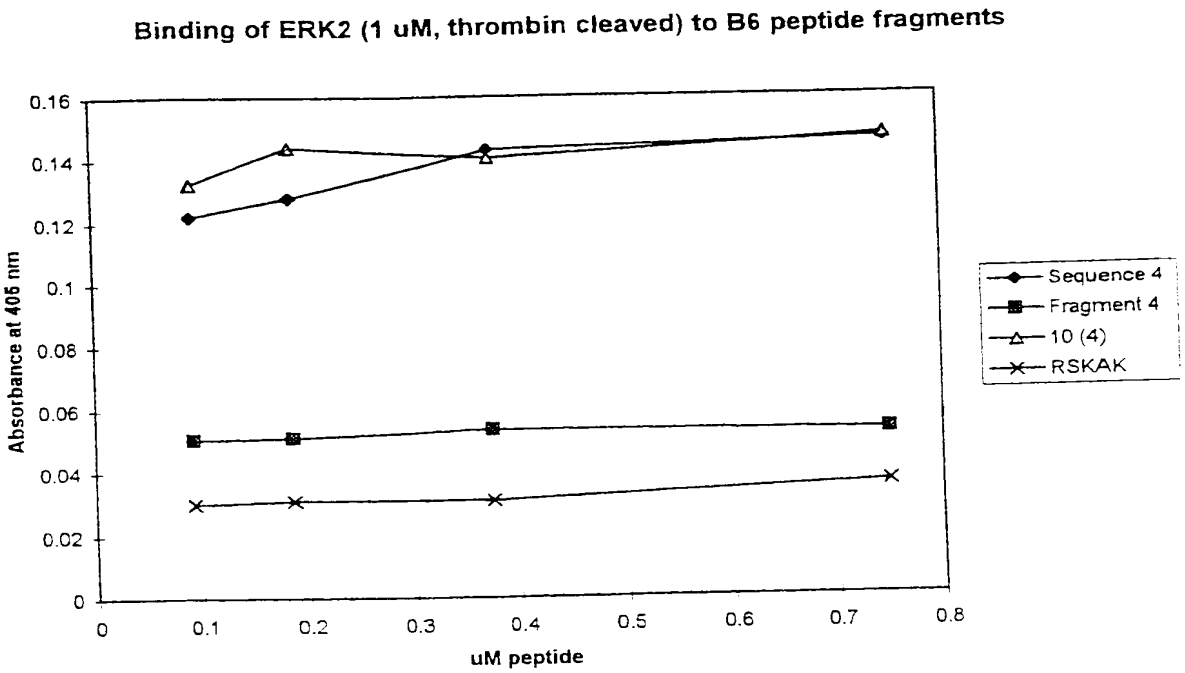
Binding of ERK2 (1 uM, thrombin cleaved) to B6 peptide fragments
Absorbance at 405 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
uM peptide
Sequence 4
Fragment 4
10 (4)
RSKAK

Figure 23

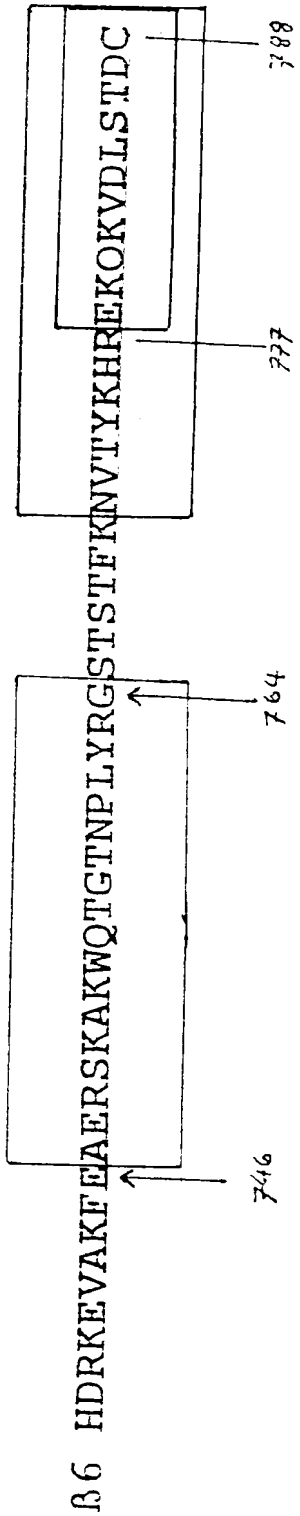
β6 HDRKEVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKOKVDLSTDC
746
764
777
788

Figure 25

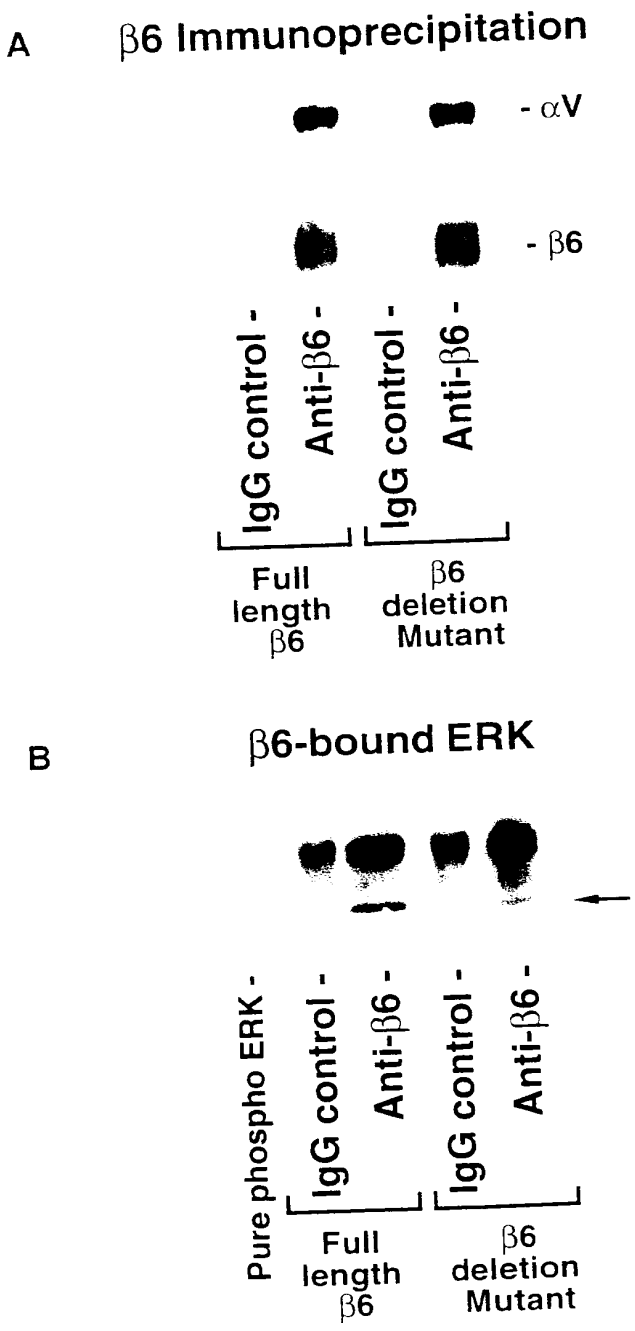
A
β6 Immunoprecipitation
- αV
- β6
IgG control -
Anti-β6 -
IgG control -
Anti-β6 -
Full length β6
β6 deletion Mutant
B
β6-bound ERK
Pure phospho ERK -
IgG control -
Anti-β6 -
IgG control -
Anti-β6 -
Full length β6
β6 deletion Mutant

Figure 27

SW480 β6 Proliferation Assay

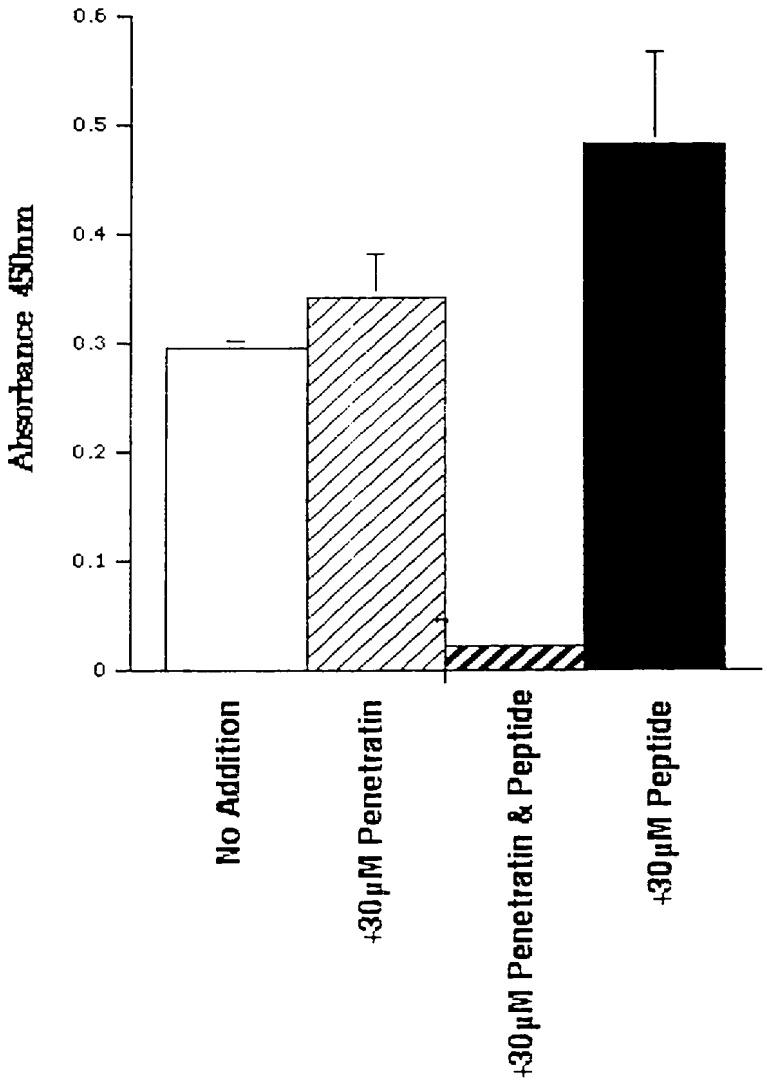

Figure 29